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[APOPTOSIS MODULATION – NEW THERAPEUTIC TARGERTS IN B CELL HEMATOLOGIC LYMPHOMAS]

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APOPTOSIS MODULATION – NEW THERAPEUTIC TARGERTS IN B CELL LYMPHOMAS

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Abstract

Cancer is a multifactorial disease resulting from the accumulation of various molecular alterations that can affect proliferation, differentiation and apoptosis. TRAIL (TNF-Related Apoptotic Inducing Ligand), also known as APO2L, can bind and interact with five different receptors: TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin. The binding of TRAIL to R1 and R2 induces apoptosis by caspase activation, while binding to anti-apoptotic receptors R3 and R4 can inhibit apoptosis and lead to chemotherapy resistance. Furthermore, the sensitivity to apoptosis is related to the level of caspase inhibitory proteins, IAP (Inhibitors of Apoptosis Proteins) such as survivin. Despite the great advances in therapy to improve the treatment of hematologic malignancies, mortality rate remains high and, consequently, the demand for more effective and specific cancer therapeutics remains a priority. The objective of this work is to study the therapeutic potential of recombinant human TRAIL (rhTRAIL) and survinin inhibitors, silibinin (SLB) and gambogic acid (GA) in B cell malignancies, specifically in chronic lymphocytic leukemia (CLL-B) and diffuse large B-cell lymphoma (DLBCL). For this purpose CLL-B (EHEB cells) and DLBCL (Farage cells) cell lines were incubated in the absence and presence of increasing concentrations of rhAPO2L/TRAIL, SLB and GA, in monotherapy. Cell viability was analyzed by trypan blue assay. Cell death was analyzed by optical microscopy (May-Grünwald-Giemsa), and flow cytometry (annexinV/propidium iodide). Expression of survivin, TRAIL ligand and its receptors was assessed by flow cytometry using monoclonal antibodies labelled with fluorescent probes. Our results show that rhTRAIL, GA and SLB, induced a decreased on cell viability in a dose, time and cell type dependent manner inducing cell death mainly by apoptosis. In EHEB cells, the IC_{50} after 24h of incubation with GA was obtained in a range of concentrations between 750 nM to 1000 nM, and of SLB with 100 µM to 150 µM. On the other hand, in FARAGE cells, IC_{50} of GA was achieved at 24h of incubation at lower

concentrations (between 300nM and 400nM), and with SLB at 48h (100µM). Although IC_{50} wasn´t obtained with rhTRAIL in both cell lines. These results may be related with the increased ratio between pro- and anti- apoptotic TRAIL receptors, the activation of intrinsic, extrinsic, or both, apoptotic pathways, and the decreased survivin expression.

Our preliminary study suggests that rhTRAIL, silibilin and gambogic acid can be used as a new therapeutic approach in the treatment of Diffuse large B-cell Lymphoma and in Chronic Lymphocytic Leukemia as singles agents, but in order to improve treatment efficacy, combination of these agents must be tested. The optimized combination may reduce toxicity and side effects, and eventually overcome treatment failure and drug resistance.

Keywords: Apoptosis**,** Chronic Lymphocytic Leukemia B, Diffuse Large B-Cell Lymphoma, rhTRAIL, Silibinin, Gambogic acid.

Resumo

O cancro é uma doença multifatorial que resulta da acumulação de várias alterações moleculares que afetam a proliferação, a diferenciação e a apoptose celular. O TRAIL (TNF-Related Apoptotic Inducing Ligand), ou APO2L, pode ligar-se e interagir com cinco tipos de recetores: TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, e a osteoprotegerina. A ligação do TRAIL aos recetores R1 e R2 induz apoptose pela ativação das caspases, enquanto que a ligação aos recetores anti-apoptóticos, R3 e R4, pode inibi-la, tornando as células resistentes à terapêutica. Além disso, a sensibilidade de uma célula à apoptose pode estar relacionada com o nível de proteínas inibidoras das caspases, as IAP (Proteínas Inibidoras da Apoptose), como a survivina. Apesar dos grandes avanços nas últimas décadas no sentido de melhorar o tratamento das neoplasias hematológicas, a taxa de mortalidade permanece elevada, pelo que a procura de terapêuticas mais eficazes e dirigidas à célula cancerígena continua uma prioridade. O objetivo deste trabalho é estudar o potencial terapêutico do rhTRAIL e dos inibidores da survinina, nomeadamente da silibinina e do ácido gambógico em tumores de células B, especificamente na Leucemia Linfocítica Crónica (LLC-B) e no Linfoma Difuso de Grandes Células B (LDGC-B). Para o efeito linhas celulares de LLC-B (as células EHEB) e de LDGB (as células FARAGE) foram incubadas na ausência e na presença de concentrações crescentes de rhTRAIL, de silibinina (SLB) e ácido gambógico (GA), em monoterapia. A proliferação e viabilidade celular foram determinadas pelo teste de exclusão do azul de tripano. A morte celular foi analisada por microscopia ótica (May-Grünwald-Giemsa), e por citometria de fluxo (AnexinaV/Iodeto de propídeo). A expressão do TRAIL ligando e dos seus recetores e da survivina, foi avaliada por citometria de fluxo, com anticorpos marcados com sondas fluorescentes. Os resultados mostram que o rhTRAIL, a silibinina e o acido gambógico, induzem uma diminuição na viabilidade celular, dependente da dose, do tempo de incubação e do tipo celular, predominantemente por apoptose. Na linha celular EHEB o IC50

foi obtido com o GA entre as concentrações de 750 nM e 1000 nM e com a SLB entre 100 uM e 150 uM, após 24 horas de incubação. Por outro lado, nas células FARAGE, o IC50 com o GA, foi obtido após 24 de incubação em doses mais baixas (entre 300 nM e 400 nM), e com a SLB após 48h com a concentração de 100 uM. Contudo IC_{50} não foi atingido em nenhuma linha celular com o rhTRAIL. Estes resultados podem dever-se ao aumento da razão entre os recetores pró- e anti- apoptóticos, à ativação da via intrínseca, da via extrínseca ou ambas, e à diminuição da expressão da survivina. O nosso estudo preliminar sugere que o rhTRAIL, a silibinina e o acido gambógico podem ser usados, em monoterapia, como novos potenciais terapêuticos no tratamento da Leucemia Linfocítica Crónica e do Linfoma Difuso de Grandes Células B. Contudo, de forma a melhorar a eficácia do tratamento a combinação deve ser testada. A combinação pode reduzir a toxicidade e os efeitos colaterais, e, eventualmente, ultrapassar o fracasso e a resistência ao tratamento.

Abbreviations List

TRAIL- TNF-related apoptosis inducing ligand

rhTRAIL- recombinant human TRAIL

DLBCL- diffuse large B-cell lymphoma

CLL-B – Chronic Lymphocytic Leukemia-B

IAP - Inhibitor of Apoptosis Proteins

GA- Gambogic Acid

SLB- Silibinin

FC- Flow cytometry

AV – Annexin V-FITC

PI – Propidium Iodide

Introduction

Apoptosis is a forcefully regulated form of cell death that is crucial for the normal development of hematopoietic cells and their equilibrium between life and death. In fact, recent progress has broadened our understanding of cancer and its underlying etiology being defective apoptosis a major causative factor for the development and progression of cancer, in the arising relapses to conventional therapy and in drug resistance[1].

In the past decade, several research have shown that the interaction of death receptors of tumour necrosis factor receptor (TNF-R) family and their ligands TNF/TNF-R, FAS/FAS Ligand and TNF-Related Apoptotic Inducing Ligand, TRAIL/TRAIL-Rs, are essential for maintaining an intact immune system surveillance against infection and cancer development[2, 3]. Since then, TRAIL was raised curiosity in scientific community because one particularity: TRAIL does not harm most of the normal cells but is capable of inducing programmed death in malignant cells [1, 4].

TRAIL or Apo-2 ligand is a type II membrane protein that exerts maximal biologic activity in its trimeric structure. It´s constituted by 281 amino acids with an intracellular N-terminus and a conserved TNF homology domain at its C-terminus. TRAIL can trigger and activate 5 different receptors of TNF family: TRAIL-R1 and TRAIL -R2, also known as death receptor, DR4 and DR5 respectively, TRAIL-R3 and TRAIL -R4, or decoy receptors, DcR1 and DcR2, and osteoprogesterin (OPG) a soluble inhibitor of receptor activator of NF-kB (RANK) ligand at low affinity. TRAIL-R1 and TRAIL-R2 are characterized by an intracellular death domain that activates the extrinsic apoptotic pathway[5]. In contrast, TRAIL-R3 and TRAIL-R4 are antagonist of apoptotic receptors, because TRAIL-R3 lacks an intracellular domain and instead has a glycosyl-phosphoinositol membrane anchor and TRAIL-R4 only have a partial intracellular death domain[5] (Figure 1).

TRAIL dead receptors can recruit the initiators caspases 8 and 10, by a homotypic interaction between death effectors domains of the adaptor molecule FAS-associated death domain (FADD) protein, and the prodomain of the initiator caspase, thereby forming the death inducing signalling complex (DISC). Within DISC procaspase-8 drives its auto activation through oligomerization and subsequently activates the downstream effectors caspase, such as caspase-3 and caspase-7, culminating in an effective apoptotic response[6]. Although in cancer cells, following death receptor binding, the level of initiator caspase activation is insufficient to induce apoptosis and the intrinsic mitochondrial pathway is necessary to amplify apoptotic signal through cleavage of BID, by caspase-8. In turn, the truncated BID activates BAX and BAK leading to the release of Cytochrome c and SMAC/DIABLO from mitochondria with subsequent induction of cell apoptosis (Figure 1)[7].

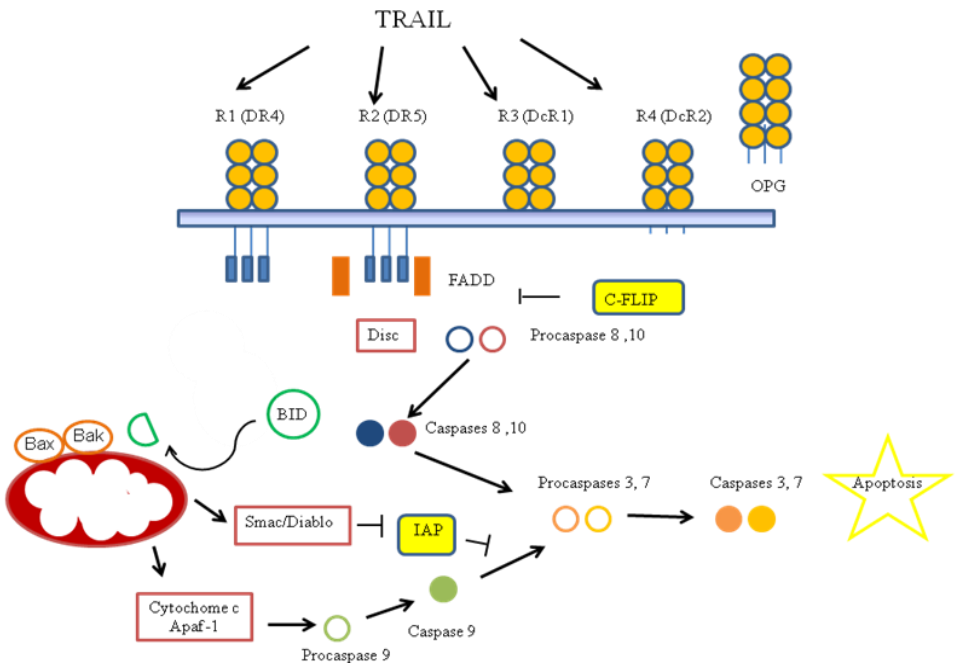

Figure 1. **Activation of intrinsic and the extrinsic apoptotic pathways by TRAIL.** Death receptor DR4 (TRAIL-R1) and DR5 (TRAIL-R2) induced extrinsic and intrinsic apoptotic pathways. Each pathway begins by caspase 8 activation. Extrinsic pathway proceeds with direct activation of caspase 3 and 7 by activated caspase 8 and 10. Intrinsic/mitochondrial pathway activation involves the cleavage of BID to activate BCL-2 family members, to depolarize mitochondrial membrane and release cytochrome c and SMAC/DIABLO. Cytochrome c interacts with APAF-1 and procaspase 9, forming caspase 9 and amplified the apoptotic process.

Although some of the tumour cells are resistant to TRAIL, different mechanisms have been described that could block TRAIL-mediated apoptosis, namely: 1) mutations at TRAIL receptors that induce loss of DR4 expression through homozygous deletion or lack of expression of death receptor on the cell surface; 2) competitive binding of TRAIL by decoy receptors TRAIL-DcR1 and TRAIL-DcR2; 3) loss of caspase-8 expression by gene methylation; 4) lack of activation of the mitochondrial pathway by overexpression of antiapoptotic proteins (BCL-2 and BCL-XL) or pro-apoptotic genes mutation (BAX); and 5) overexpression of c-FLIP and survivin[8, 9].

Survivin is a member of the IAP (inhibitor of apoptosis proteins) family of proteins, of which nine members are known: NAIP (neuronal apoptosis inhibitor protein) or BIRC1; c-IAP-1

(cellular-IAP1) or BIRC2; c-IAP-2 (cellular–IAP2) or BIRC3; XIAP (X chromosome-linked IAP) or BIRC4; survivin or BIRC5; apollon (ubiquitin-conjugating BIR domain enzyme apollon) or BIRC6, and linin or KIAP or BIRC 9. Although expressed at high levels during fetal development, survivin is rarely expressed in normal healthy adult cells. It is however upregulated in the majority of cancer cells[10]. This molecule is the smallest member of the IAP family, containing 142 amino acid residues. Besides its function as a potent inhibitor of apoptosis namely thought interactions with capases 9, 3 and 7, it is also involved in cell proliferation, angiogenesis, resistance to multiple types of anticancer therapy, and has consistently been implicated as a negative prognostic factor in patients with solid tumours and lymphoid cancers.[11] Besides that, inhibition of survivin becomes a target for cancer treatment[12]. Silibinin (SLB), a flavonoid derived from *Silybum marianum*, is a survivin inhibitor that is capable of blocking cell cycle, apoptosis, preventing angiogenesis and tumor invasion[13]. Gambogic acid (GA) is the principal active ingredient of gamboges which is the resin from various *Garcinia* species, and has various biological effects, such as anti-inflammatory, analgesic and anti-pyretic, as well as anti-cancer activities by interfering with surivivin.[12].

Chronic Lymphocytic Leukemia B (CLL-B), the most common leukaemia in western countries, is a lymphoid neoplasia of B-cells that is characterized by a low proliferation rate and impaired apoptosis[14]. The disease has an indolent course but, usually is resistant to conventional therapies.[15] On the other hand, Diffuse large B-cell lymphoma (DLBCL), is a common type of non-Hodgkin lymphoma, accounting for about 25 to 30% of all cases of lymphomas in the western countries[16]. It is a clinically aggressive lymphoma with an elevated rate of cellular proliferation and the patients have a five-year survival rate of 50%.[15]

Despite the great advances in therapy to improve the treatment of hematologic malignancies, mortality rate remains high and, consequently, the demand for more effective and specific cancer therapeutics to remains a priority.

In this context, the aim of this work is to analyse the therapeutic efficacy of a recombinant TRAIL (rhTRAIL) and two natural inhibitors of survivin, silibinin and gambogic acid, in Chronic Lymphocytic Leukemia B-cell (CLL-B), and in Diffuse Large B-cell Lymphoma (DLBCL).

Materials and Methods

Cell culture

In this study we used two hematological neoplasic cell lines, both from lymphoid origin. The CLL-B cell line, EHEB cells, isolated from the peripheral blood of a 69-year old woman, was provided by German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. The diffused large B cell lymphoma (DLBCL) cell line, the FARAGE cells, was obtained from a lymph node biopsy from a patient with diffuse large B cell lymphoma, purchased from American Type Culture Collection, (ATCC). Cell lines were routinely grown in RPMI-1640 medium (L-glutamine 2 mM, HEPES-Na 25 mM, penicillin 100 U/mL and streptomycin 100 µg/mL) supplemented with 20% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were cultured at initial density of $0{,}5x10^6$ cells/ml, during 72 hours, in absence and in presence of increasing concentrations of rhTRAIL, silibilin and gambogic acid.

Cell viability assays

To analyze the therapeutic effect of rhTRAIL, cells were incubated in absence (control) and in presence of different rhTRAIL concentrations (between 10 and 750 $ng.ml^{-1}$), silibilin (SLB)

(between 10µM and 150 µM), and gambogic acid (GA) (between 50 nM and 1000 nM), in monotherapy. Cell proliferation was accessed by cell counting in a neubauer chamber and cell viability was estimated by trypan blue exclusion each 24h during 72h. The trypan blue solution is a vital stain that is not absorbed by healthy viable cells. When cells are damaged or dead, trypan blue can enter the cell allowing dead cells to be counted in a neubauer chamber.

Morphological and flow cytometry assessment of cell death

Morphological aspects of cell death were evaluated by cell smears stained with May-Grünwald-Giemsa. Cells incubated with and without drugs, during 48h, were transferred to slides and stained. Cell death was evaluated by analysing morphological characteristics under light microscopy, using a Nikon Eclipse 80i equipped with a Nikon Digital Camera DXm 1200F.

Cell death was also analysed by flow cytometry using annexin V-FITC (AV) and propidium iodide (PI) incorporation. During apoptosis, phosphatidylserine, a phospholipid located in inner surface of plasma membrane, goes to outer surface allowing the binding of AV. PI is a DNA intercalator that enters cells having damaged membrane (necrotic cells). Then, it is possible to distinguish apoptotic cells from viable and necrotic cells.

Briefly, cells were washed with ice-cold phosphate buffer (PBS) (centrifuged at 1 000 xg for 5 min), ressuspended in 100 µL of binding buffer and incubated with 5 µL of AV-FITC solution and 5 µL of PI solution for 15 min in the dark. After incubation time, cells were diluted in 400 µL of ice-cold binding buffer, and analyzed by flow cytometry. Results are expressed in percentage (%) ± SD of at least 3 independent experiments. Flow cytometry analysis was performed using a six-parameter, four-color FACSCalibur^{TM} flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 nW argon laser. For each assay 1 x 10^6

cells were used and at least 10.000 events were collected by acquisition using CellQuest software (Becton Dickinson) and analyzed using Paint-a-gate software (Becton Dickinson).

Expression of apoptosis related proteins by flow cytometry

To determine TRAIL ligand and receptors surface expression, $1x10^6$ cells cultured in the absence (control) and presence of rhTRAIL, SLB and GA, were incubated with 1 µg of anti-TRAIL-R1 labeled with phycoerythrin (PE), anti-TRAIL-R2, anti-TRAIL-R3, anti-TRAIL-R4 (R&D Systems) or anti-TRAIL Ligand-PE (BD Bioscience) monoclonal antibodies, during 15 minutes in the darkness. Then, cells were washed with PBS thought centrifugation, during 5 min, ressuspended in 300 µl of PBS and analyzed by flow cytometry. To assess the cytoplasmatic expression of survivin, cells were stained with monoclonal antibody anti-survivin-PE (R&D Systems), according with manufactured protocols. Briefly, cells were incubated with 100 µl of fix solution (IntraCell; Immunostep) for 15 min and washed by centrifugation at 300 xg for 5 min. Then, cells were permeabilized and incubated for 15 min with 100 µl of permeabilization solution (IntraCell; Immunostep) and 1 µg of anti-survivin-PE antibody. After a wash step, cells were analyzed by flow cytometry. The levels of cellular fluorescence, proportional to the concentration of these proteins in each cell, were measured by flow cytometry and results were plotted in Mean Fluorescence Intensity (MFI) arbitrary units. This value represents the medium fluorescence intensity detected in cells, which is proportional to the number of molecules labeled by antibody.

Statistical analysis

Data are expressed as mean±SD obtained from independent assays, each one performed in duplicate or triplicate. Differences between data sets were evaluated by Student's *t*-test. A *p* value <0,05 was considered as statistically significant.

Results

Cell viability analysis

Initially, we investigate the influence of rhTRAIL in viability of EHEB and FARAGE cell lines (concentrations ranging from 10 ng/mL to 750 ng/mL). As we can observe in Figure 2, rhTRAIL induced a decreased on cell viability in a dose, and time dependent manner, in both cell lines. Although rhTRAIL show a cytotoxic effect, IC_{50} wasn't achieved in any cell line.

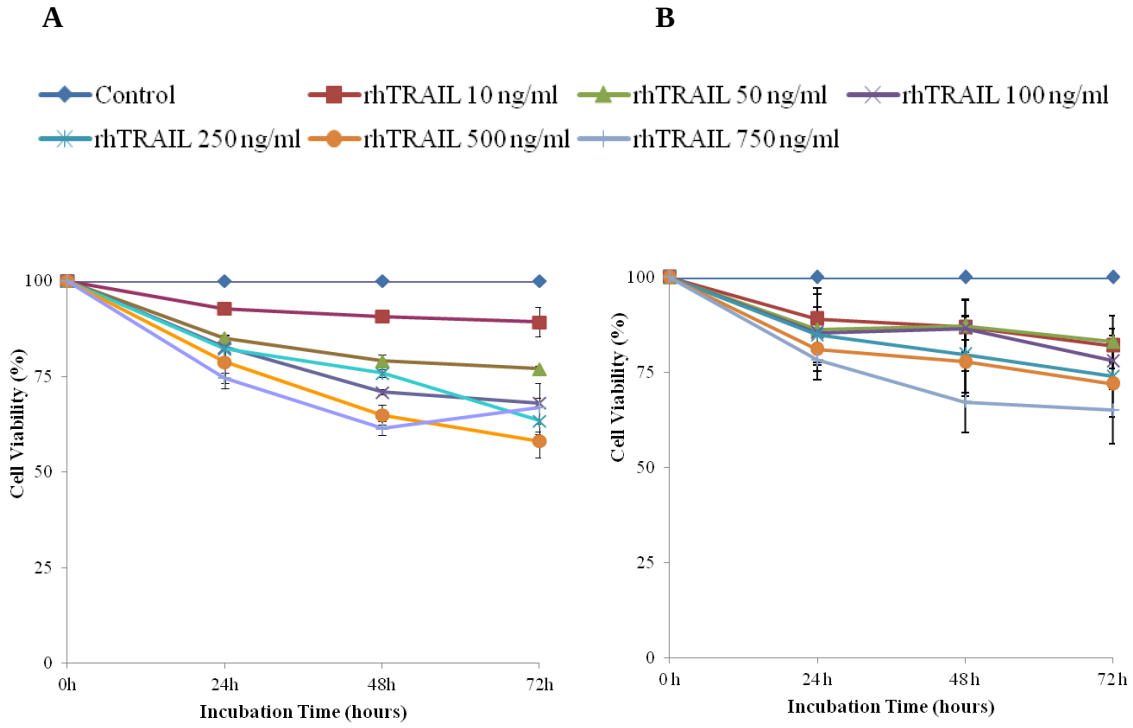

Figure 2. Dose response curves of rhTRAIL in EHEB (A) and Farage cell lines (B). Cell viability was analysed each 24h during 72 h after incubation with different rhTRAIL concentrations as it is represented in figure. It is represented the effect of rhTRAIL in cell viability of EHEB (A), and FARAGE cell lines (B). Data is expressed in percentage (%) normalized to the control and represents the mean± SD of at least 3 independent experiments.

Then, cells are treated with two natural inhibitors of survivin, Silibilin (SLB) and Gambogic Acid (GA), as represented in Figures 3 and 4. When cells are treated with SLB we observe also a decreased in cell viability in a dose and time dependent manner, more dose dependent in EHEB cells, and time dependent in FARAGE cell line. However, the results obtained are also cell type dependent (Figure 3). In fact, Figure 3A show that, besides in EHEB cells the

IC_{50} was achieved earlier, after 24h of incubation, and with 100 µM of drug concentration, the maximal cytotoxic at 72 h is obtained with 150 µM. By contrast, in FARAGE cells, the IC_{50} of SLB is achieved only after 48h of incubation with 100 µM of the drug (Figure 3B), but to obtain the maximal cytotoxic effect (100%), lower SLB concentration is needed in this cell line (100 µM). In fact SLB is more effective in FARAGE cell line than in EHEB.

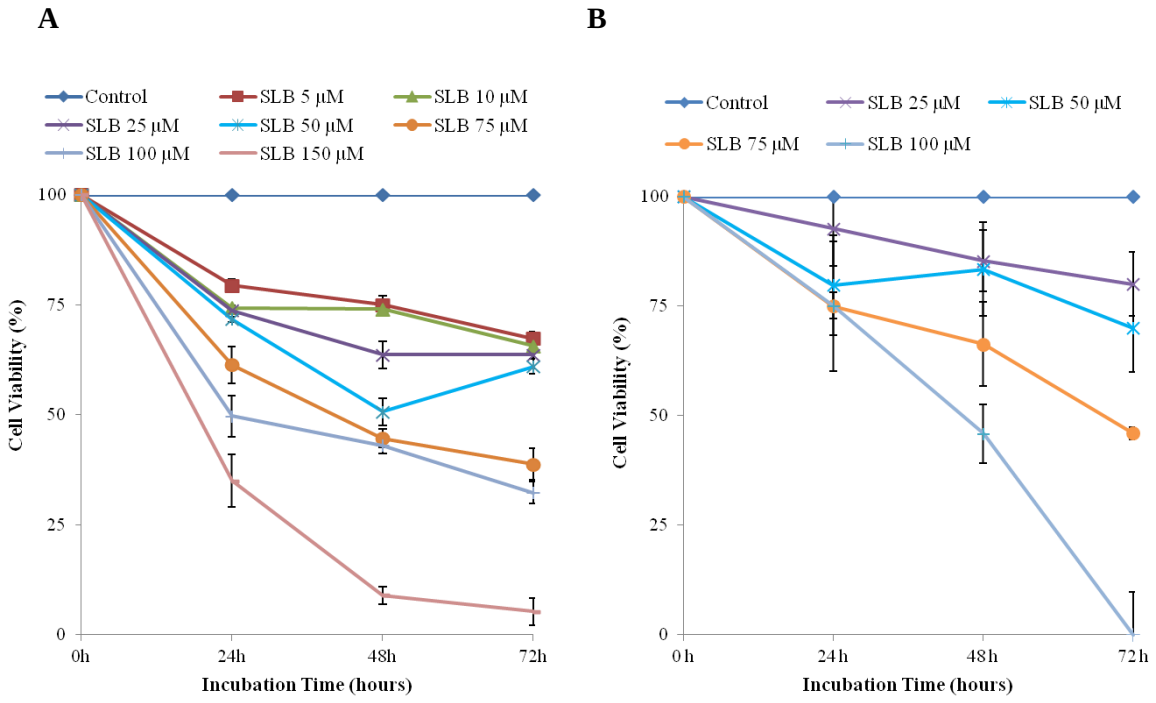

Figure 3. Dose response curves of silibinin (SLB) in EHEB cell line (A) and in Farage cell line (B). Cell viability was analysed each 24h during 72h after incubation with different concentrations of SLB as it is represented in figure.. It is represented the effect of SLB in cell viability of EHEB (A) and FARAGE cell lines (B). Data is expressed in percentage (%) normalized to control and represents the mean± SD of at least 3 independent experiments.

Similarly to the results obtained with SLB, in EHEB and FARAGE cells treated with GA, we observed a decreased in cell viability in a dose, time and cell type dependent manner. As we can see in Figure 4, besides the IC_{50} is achieved in both cells lines at 24h, it is obtained with different drug concentrations. In EHEB cells the IC_{50} is between 500 nM and 750 nM (Figure 4A) and in FARAGE cell line, the IC_{50} is achieved at lower concentration (400 nM) (Figure

4.B). As occur with SLB, GA seems to be more effective in FARAGE cell line than in EHEB cell line.

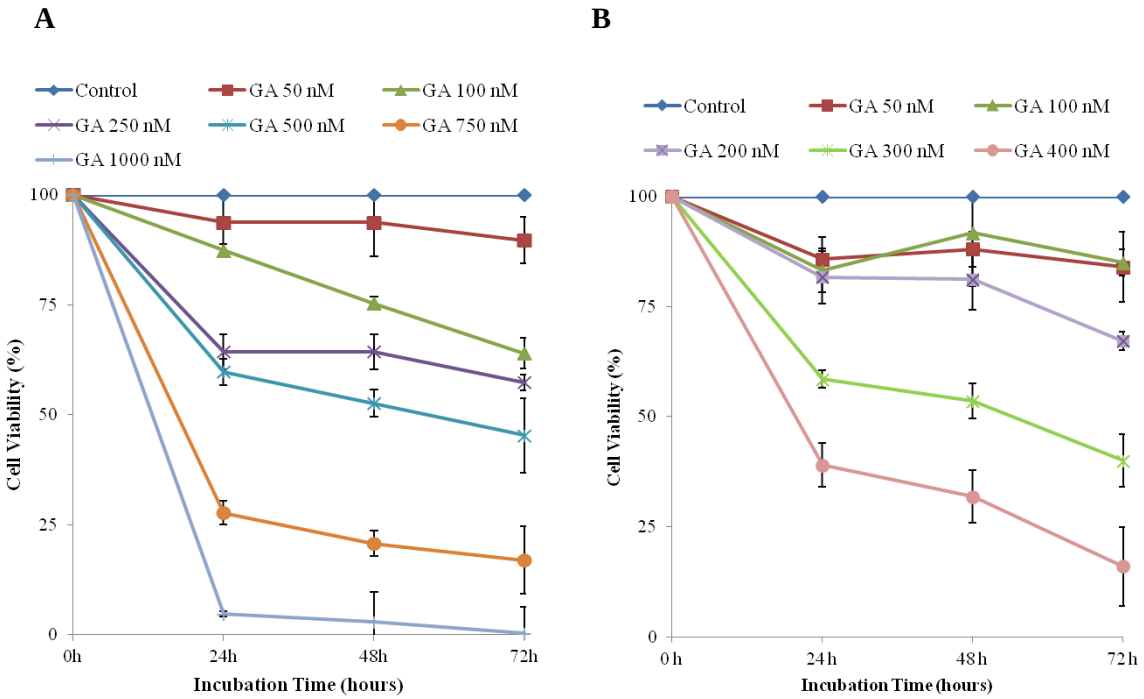

Figure 4. Dose response curves of Gambogic Acid (GA) in EHEB (A) and in Farage cell lines (B). Cell viability was analysed each 24h during 72h of incubation with different concentrations of GA as represented in figure. It is represented the effect of GA in cell density and viability of EHEB (A) and in FARAGE cell lines (B). Data is expressed in percentage (%) normalized to control and represents the mean± SD of at least 3 independent experiments.

Cell death analysis

To analyzed cell death induced by these compounds, we analyzed the morphological aspects of EHEB and FARAGE cells lines after 48h of incubation, with different concentrations of rhTRAIL, SLB and GA, as represented in Figure 5. As we can observed, these survivin inhibitors induced cell death mainly by apoptosis. In fact, EHEB and FARAGE cells lines treated with these compounds exhibited morphological characteristics of apoptosis, namely nuclear fragmentation, cellular contraction, blebbing and apoptotic bodies.

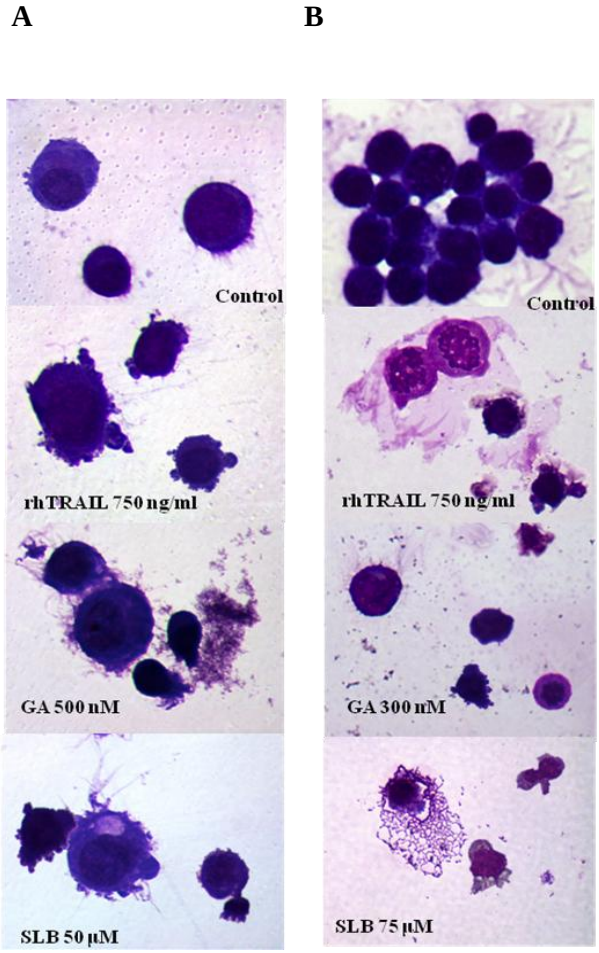

Figure 5. Cell death analysis by optical morphology. Morphological aspects of EHEB cell line (A) and FARAGE cell line (B). Cells were grown in the absence (control) and in the presence of SLB, GA and rhTRAIL in the concentrations indicated in the figure. After, the stained smears were analysed using a light microscope. Amplification: 500x.

To confirm the cytotoxic effect of rhTRAIL, GA and SLB in EHEH and FARAGE cell lines, observed in the morphological studies, cell death was also evaluated by flow cytometry using annexin V/PI incorporation after 48h of incubation, as indicated in Figure 6. Results indicate that these drugs induce predominantly apoptosis since we do not observe PI incorporation in cells treated with rhTRAIL, SLB and GA. Moreover, as we can see in Figure 6 when EHEB cells were incubated with 500 ng/ml rhTRAIL, 500 nM GA and 50 µM of SLB for 48h, we observed an increase of 41% ± 0,7%, 49% ± 0% and 46% ± 0,7% in apoptotic cells, respectively. Moreover in FARAGE cells we observed similar results. When these cells were

cultured in the presence rhTRAIL (750 ng/ml), GA (300 nM) and SLB (75 µM) an identical increase in apoptotic cells of 40% ± 0,6 %, 47% ± 1% and a 39% ± 7%, respectively, was observed.

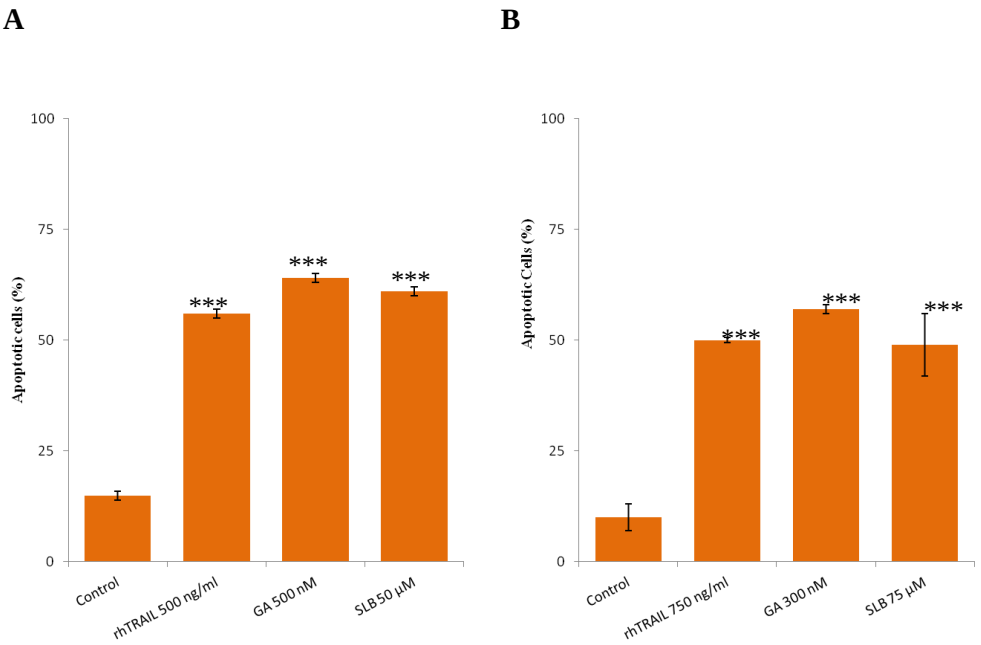

Figure 6. Cell death analysis by flow cytometry. In (A), it is represented the results obtained in EHEB cell line and in (B) in FARAGE cells. Both cells lines were incubated during 48h in absence (control) and in presence of rhTRAIL, SLB and GA, in concentrations indicated in figure. Cell death was detected by annexinV and propidium iodide staining and analyzed by flow cytometry as refereed in material and methods. Results are expressed in % ± SD and represent the mean± SD of at least 3 independent experiments. ***p<0,001

Expression of apoptosis related proteins by flow cytometry

To analysed the influence of rhTRAIL, GA acid and SLB in TRAIL signalling pathway, we have studied the expression levels of TRAIL Ligand, TRAIL receptors and survivin by flow cytometry, in basal conditions and after therapy.

In basal conditions, as we can see in Figure 7, the expression of TRAIL ligand is higher 3-fold in EHEB (63 ± 6) than in FARAGE (21 ± 3) cells. However, when we evaluated the ratio between TRAIL pro- and anti- apoptotic receptors (R1+R2)/(R3+R4) we observe the

opposite. In fact, FARAGE cells (1,1 ± 0,05) show an increased ratio between pro- and anti-apoptotic TRAIL receptors about 1,66-fold compared with EHEB cells (0,7 ±0,07), meaning that FARAGE cells have more pro-apoptotic receptors. Moreover, survivin expression show that, EHEB cells have 2,6-fold higher expression levels of surviving (72 ± 0,7), compared to FARAGE cells (27 ± 2).

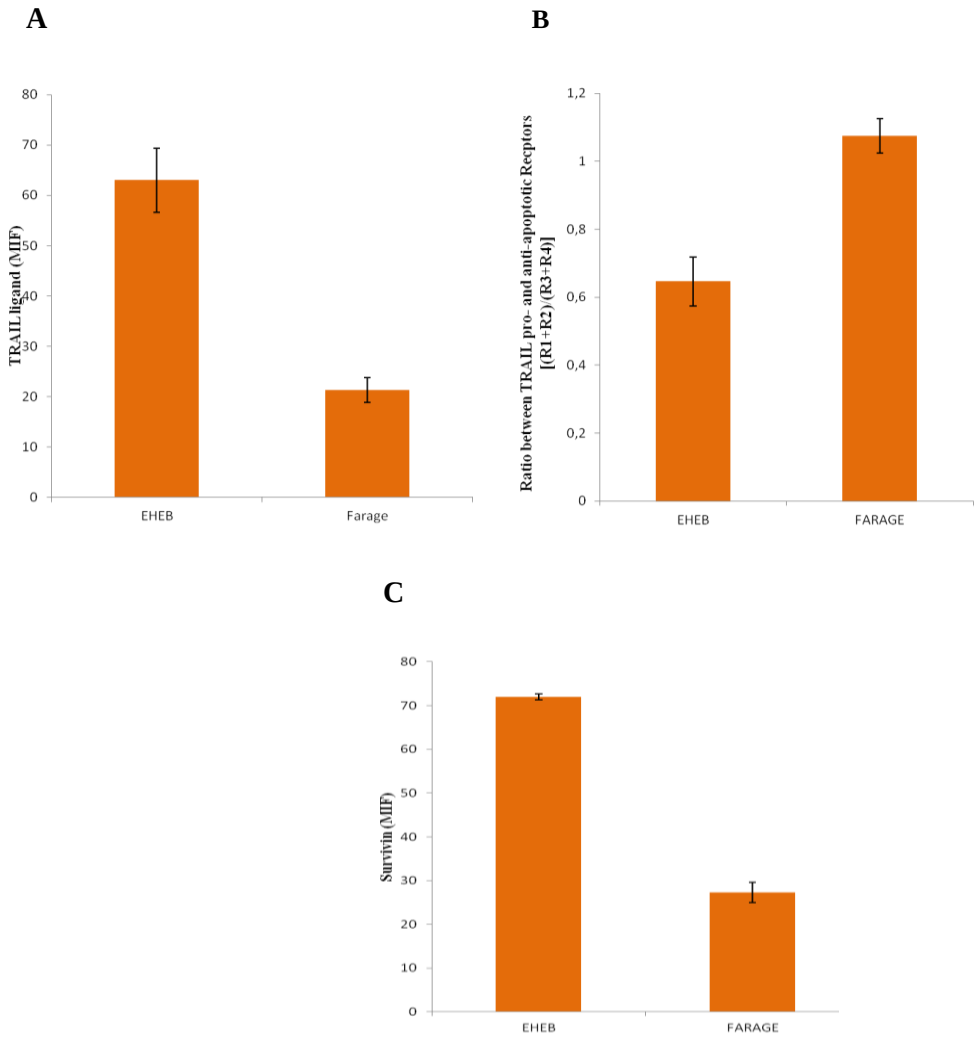

Figure 7. Evaluation of Basal expression levels of TRAIL Ligand (A), ratio between pro-apoptotic and anti-apoptotic TRAIL receptors (R1+R2/R3+R4) (B) and survivin (C) in EHEB and FARAGE cell lines by flow cytometry. Results are expressed in mean intensity of fluorescence (MIF) and represent the mean± SD of at least 3 independent experiments.

As observed in Figure 8, when EHEB and FARAGE cells lines were cultured in presence of rhTRAIL, GA acid and SLB, during 48 h of incubation, we observed an increase in TRAIL

ligand expression. In fact, in EHEB cells the observed increase is 1,44-fold with rhTRAIL (500 ng/ml), 1,17-fold with GA (500nM) and 1,38-fold with SLB (50 µM). In FARAGE cells, the increase in TRAIL ligand expression levels were more pronounced and is about 1,48-fold with rhTRAIL (500 ng/ml), 1,39-fold with GA (300nM), and 2,17-fold with SLB (75 µM).

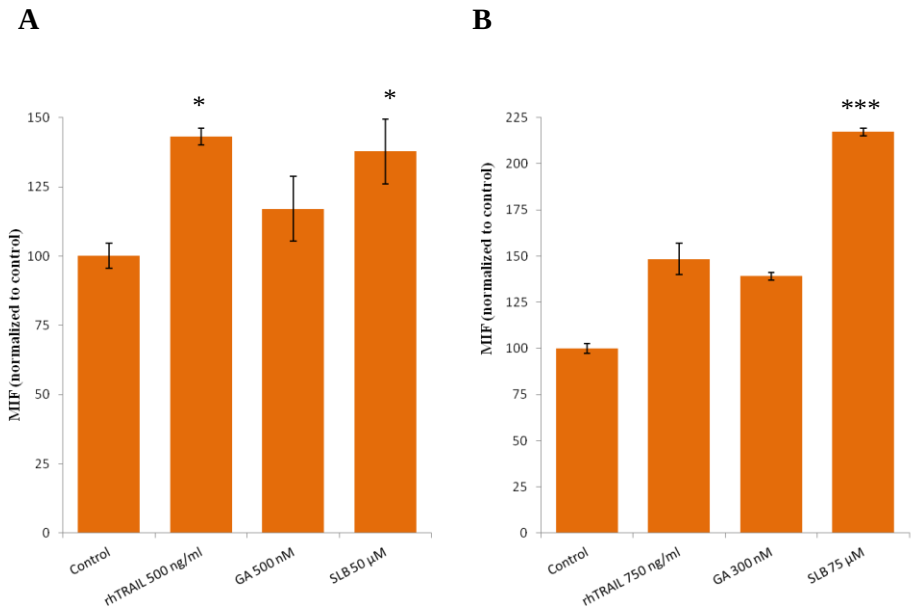

Figure 8. Expression of TRAIL Ligand by flow cytometry. Figure represents the results obtained by flow cytometry when EHEB cell line (A) and FARAGE cell line (B) were 48h incubated in absence (control) and in presence of drugs, as it is indicated in the figure. Results are expressed in mean intensity of fluorescence (MIF) normalized to control and represent the mean± SD of at least 3 independent experiments. *p<0,05, ***p<0,001

Next, we analysed the ratio between proapoptotic (R1 and R2) and antiapoptotic (R3 and R4) TRAIL receptors, Figure 9. As observed in basal conditions the ratio is higher in FARAGE cell line (1,1 ± 0,05) than in EHEB cell line (0,7 ±0,07), meaning that FARAGE cells have a higher amount of proapoptotic receptors compared with antiapoptotic than EHEB cells. However, when EHEB cells are treated with rhTRAIL (500 ng/ml) an increase of 11% is observed (Figure 9A), while in FARAGE cells this compound induced a decreased of 27% in this ratio compared with control (Figure 9B). When cells were treated with GA the R1+R2/R3+R4 ratio show an increase of 0,5% in EHEB cell line and of 16% in FARAGE

cell line (Figure 9). Moreover, EHEB cells incubated with SLB show an increased of R1+R2/R3+R4 ratio about 88% (Figure 9A), while in FARAGE a decreased of 15% is observed (Figure 9B).

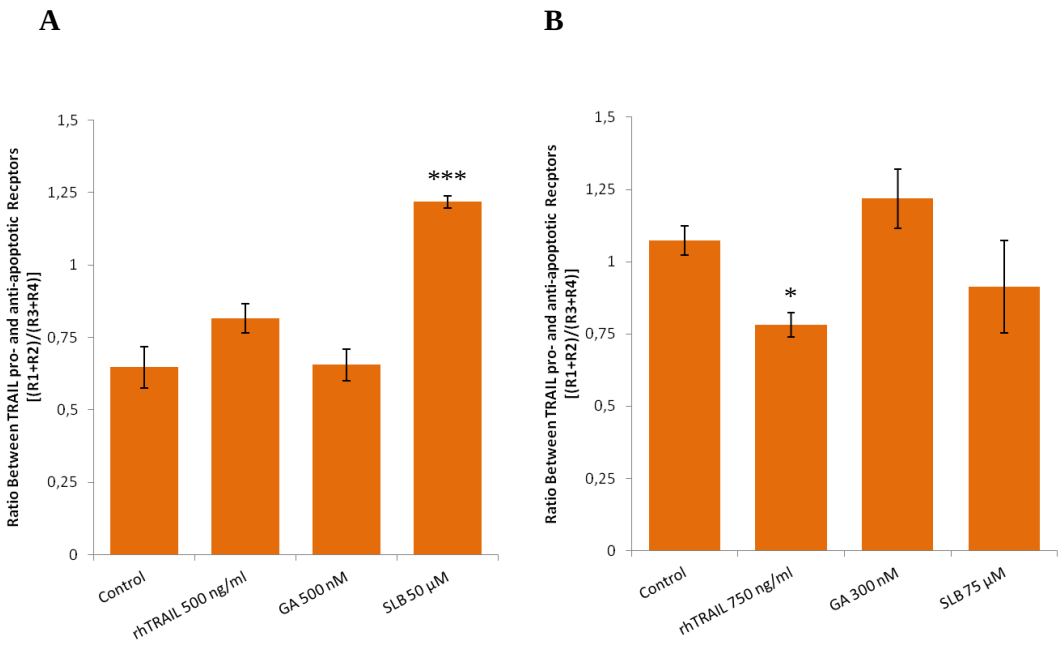

Figure 9. Evaluation of the ratio between pro-apoptotic and anti-apoptotic TRAIL receptors by flow cytometry. The expression of TRAIL-R1, R2, R3 and R4 were evaluated by flow cytometry, as described in material and methods, and the ratio (R1+R2)/(R3+R4) was determined in EHEB cell line (A) and FARAGE cell line (B) in absence (control) and in presence of drugs in concentration indicated in figure, during 48h. Results are expressed in mean intensity of fluorescence (MIF) and represent the mean± SD of at least 3 independent experiments. *p<0,05, ***p<0,001

Finally, we analysed the expression levels of survivin as represented in Figure 10. As we can observed, in EHEB cells treated with rhTRAIL (500 ng/ml) we observed a decreased of 18% in survivin expression. When EHEB cells were treated with GA (500 nM) and with SLB (50 µM) a decrease of 14% and 4%, respectively, was observed. However in FARAGE cells treatment with rhTRAIL and survivin inhibitors induced a small increase in survivin expression, respectively, 5% with rhTRAIL (750 ng/ml), 2% with GA (300 nM), and 8% with SLB (75 µM).

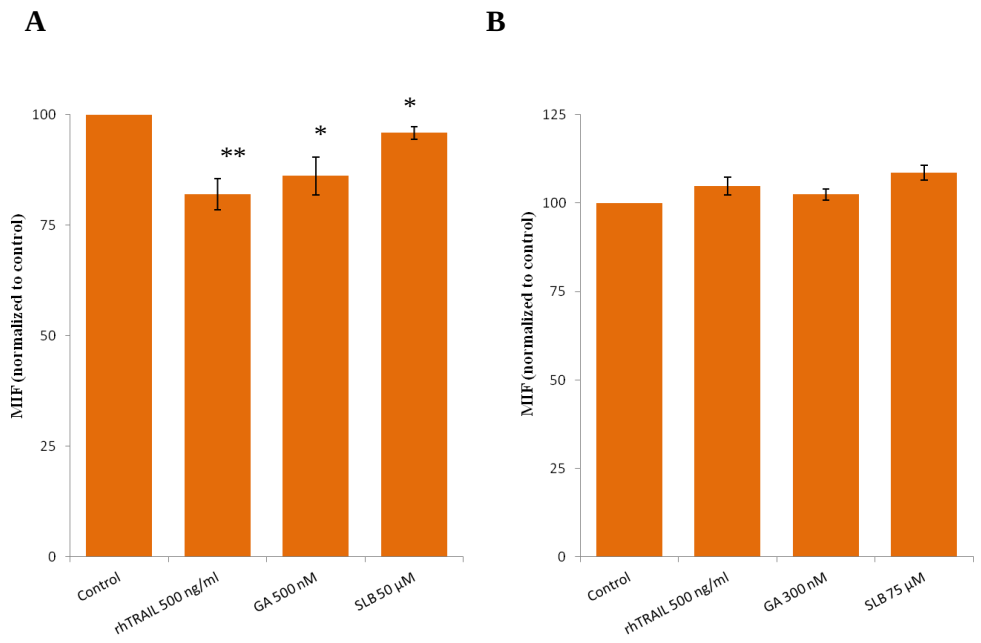

Figure 10. Analysis of the expression of survivin by flow cytometry. Figure represents the results obtained by flow cytometry when EHEB cell line (A) and FARAGE cell line (B) were incubated in the absence (control) and in the presence of drugs, as indicated in figure, for 48h. Results are expressed in mean intensity of fluorescence (MIF) normalized to control and represent the mean± SD of at least 3 independent experiments. *p<0,05, **p<0,01

Discusssion

Aberrant or deregulated apoptosis is a fundamental event in human malignancies, namely in lymphoid cancers, and therefore presents considerable interest for therapeutic intervention. Virtually all cytotoxic drugs and radiotherapy programs induce apoptosis in tumours cells but resistance to apoptosis is associated with treatment failure and frequent relapses[17]. Despite the significant increased of survival associated with the conventional anticancer therapies, their side effects are very severe debilitating the patient. Moreover, recurrences are frequent and development of resistance determines the treatment failure. The focus of drug development in cancer has moved from cytotoxic compounds to specific molecular targets [8]. There are multiple lines of evidence suggesting that TRAIL pathway could be a promising approach for cancer treatment based on the fact that this member of the tumor necrosis factor

family induces apoptosis in the majority of malignant cells while normal cells are resistant [4]. In fact, almost all cancer cells have a higher amount of pro-apoptotic receptors (R1/DR4 and R2/DR5) than normal cells [7, 18] On the other hand normal cells have higher levels of anti-apoptotic TRAIL Receptors (R3/DcR1 and R4/DcR2)

Several recombinant TRAIL, rhTRAIL, and monoclonal antibodies agonists of TRAIL receptors have been produced and tested in many diseases especially in solid tumors and some subtypes of lymphomas[6], but few studies have been done in DLBCL and CLL-B[1].

Our results shows that rhTRAIL induced a decrease in EHEH and FARAGE cells viability inducing cell death, in a time and dose dependent manner, although for both cells lines the IC_{50} wasn´t achieved. However, cell death analysis shown the presence of apoptosis at 48h and this may be due the fact that tripan blue assay don´t detect earlier apoptosis. These results are in agreement with previously experiments in Acute Lymphoblastic Leukemia (ALL), cell line CEM, (IC_{50} was achieved after 72h of incubation with 1000ng/ml) and in Acute Promyelocytic Leukemia cell line, HL-60 cells (IC_{50} was obtained with 200-250ng/ml at 48h) [19, 20]. Besides that, identical results using other recombinant TRAIL were found by Plasilova (2002) in HL-60 cell lines, by Rezacova (2008) in MOLT-4 cells, and by Jacquemin (2012) in diffused large B-cell lymphoma cell line (SUDHL4 cells). [21-23] However, not all hematologic cell lines are sensitive to rhTRAIL in the some way, suggesting that the cytotoxic effect of TRAIL is dependent on the cells characteristics. For example in K-562 cells, a Chronic Myelogenous Leukemia in Blast Crisis cell line, rhTRAIL does not induce a significant cytotoxic effect [19].

Furthermore, it was found that, although TRAIL induces apoptosis rather in cancer cells than in normal cells, some types of cancers are insensitivity to TRAIL treatment. Thus, various mechanisms have been associated with resistance to this ligand, namely the levels of

apoptotic *vs* anti-apoptotic TRAIL-receptors[20], and the increased expression of anti-apoptotic proteins such as survivin[24, 25].

Survivin, is a member of IAP that regulates two essential cellular processes: inhibits apoptosis and promotes cell proliferation. This molecule has some specific characteristics that make it unique, being high expressed during fetal development, but not in healthy adult tissues, and upregulated in cancer cells. Because of these characteristics, survivin is currently attracting considerable interest both as a potential cancer biomarker[26] and as a new target for cancer treatment[27]. Many strategies to inhibit survivin are being developed such as: binding to survivin promoter (e.g. YM155), by inhibiting protein translation (e.g. antisense oligonucleotides and siRNA) and by interfering with survivin function or thought natural compounds namely GA and SLB that inhibits survivin[10], [28].

In this study, the two inhibitors of survivin, GA and SLB show a decreased in cell viability in dose time dependent manner in both cells lines, more dose dependent in EHEB, and time dependent in FARAGE cells, treated with SLB. However GA and SLB seems to be more effective is FARAGE than in EHEB. This may be correlated with different expression of survivin. In fact, as is observed in Figure 7, FARAGE cell line has a lower basal expression of survivin, which could contribute to the higher sensibility to apoptosis induced by the survivin inhibitors.

Our results show that rhTRAIL, SLB and GA, induced cell death predominantly by apoptosis as confirmed by morphological studies (optical microscopy) showing the presence of apoptotic characteristic (nuclear fragmentation, cellular contraction, blebbing and apoptotic bodies) and by flow cytometry using the double staining with annexinV and propidium iodide.

In attempt to explain the previous results we studied the ratio between pro-apoptotic (R1+R2) and antiapoptotic (R3+R4) TRAIL receptors, to see if there is any correlation between the

differential TRAIL receptors expression and the sensitivity to cell death induced by TRAIL, SLB and GA. In control cells the ratio is higher in FARAGE than in EHEB cells, that mean that FARAGE cell have more pro apoptotic receptors than EHEB cells, which could explain the higher decrease in cell viability obtained in FARAGE cells after 72 hours of treatment with lower doses of rhTRAIL (10ng/ml), compared to EHEB. In fact we can extrapolate, that FARAGE cells in basal conditions can be more sensitive to rhTRAIL than EHEB cells. However, in EHEB cells, we saw an increase of the ratio in the presence of the three drugs more pronounced with SLB (88%) than with rhTRAIL and GA (11% and 0,5%, respectively). By contrary, in FARAGE cells treated with rhTRAIL, SLB and GA, a decrease in the ratio between pro and anti-apoptotic TRAIL-Rs is observed. Moreover other apoptotic pathways and/or mechanisms may be involved.

Loeder *et al.* (2009), also show the interaction between IAPS and the increased of TRAIL receptors using three CLL-B cell lines, MEC1, JVM2 and JVM3 cells. The authors show that these cells have high membrane expression of agonistic R2, whereas R3 and R4 receptors were expressed in much lower levels when cells were incubated with XIAP (a member of IAPs) [25]. XIAO *et al.* (2011), using two cell lines of CLL-B (BJAB and I-83) show an increased of R1 and R2 expression after treatment with a recombinant human soluble TRAIL[29] .

Besides that, the expression levels of survivin could also influenced the therapeutic efficacy of the compounds used in this study. In fact, FARAGE cell line has a lower basal expression of survivin, which could contribute to the higher sensibility to apoptosis induced by survivin inhibitors, SLB and GA, in dose response. However, EHEB cell line treated with rhTRAIL, GA and SLB, there was a decrease in the expression levels of survivin than those obtained in FARAGE cells treated with the drugs. These results may explain the most pronounced efficacy of these drugs in EHEB cell line at 48 hours. Although, is reasonable to think that

SLB and GA, in FARAGE and in EHEB cell lines, can interact with survivin not only through the inhibition of its expression but also through the lack of function. More experiments must be done in order to confirm this theory.

A study using a bladder transitional cell papilloma cell line (RT4 cell line) show that silibilin target survivin expression [30], and another study used human glioma cells got the same result [31]. GA in some studies has been shown to be able to induce apoptosis in multiple myeloma (RPMI-8226 cell line)[32] in human myeloid leukemia (KBM-5 cell line) and human embryonic kidney (A293 cell line)[33].

Despite the previous results, a few more things must be done, in order to understanding these interactions perfectly, such as: the correct interaction mechanism of survivin with others IAPS, caspase 9 and TRAIL; the exact mechanism(s) of inhibition of surviving by GA and SLB; the potential use of this drugs in combination with conventional therapy; and the influence of schedule drug administration.

Because IAP inhibitors and recombinant TRAIL are currently under evaluation in early clinical trials it is crucial to identify those cancers that may benefit from these new treatment options. Our preliminary study suggests that rhTRAIL, silibilin and gambogic acid can be used as a new therapeutic approach in the treatment of Diffuse large B-cell Lymphoma and in Chronic Lymphocytic Leukemia as singles agents, but in order to improve de efficacy of treatment a combination of these agents must be tested. The optimized combination may reduce toxicity and side effects, and overcome eventually drug resistance.

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